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Classical activation domains within DNA-bound eukaryotic transcription factors make weak interactions with coactivator complexes, such as Mediator, to stimulate transcription. How these interactions stimulate transcription, however, is unknown. The activation of reporter genes by artificial fusion of Mediator subunits to DNA binding domains that bind to their promoters has been cited as evidence that the primary role of activators is simply to recruit Mediator. We have identified potent classical transcriptional activation domains in the C termini of several tail module subunits of *Saccharomyces cerevisiae*, *Candida albicans*, and *Candida dubliniensis* Mediator, while their N-terminal domains are necessary and sufficient for their incorporation into Mediator but do not possess the ability to activate transcription when fused to a DNA binding domain. This suggests that Mediator fusion proteins actually are functioning in a manner similar to that of a classical DNA-bound activator rather than just recruiting Mediator. Our finding that deletion of the activation domains of *S. cerevisiae* Med2 and Med3, as well as *C. dubliniensis* Tlo1 (a Med2 ortholog), impairs the induction of certain genes shows these domains function at native promoters. Activation domains within coactivators are likely an important feature of these complexes and one that may have been uniquely leveraged by a common fungal pathogen.

Despite years of study, the mechanism by which DNA-bound transcriptional activators communicate this information to the core transcription machinery in eukaryotes is unknown. Classical eukaryotic transcriptional activation domains (TADs) typically are found in sequence-specific DNA-bound transcription factors that target coactivators (1), such as Mediator (2–4), through weak interactions to stimulate the activity of the core transcription machinery. The classical TAD is characterized by lack of defined structure, and its weak interactions are thought to facilitate the TAD touching multiple coactivator and core transcription machinery targets (5). The 20+ subunit Mediator coactivator complex is a critical functional/physical intermediary between DNA-bound activators and the general transcription machinery in all eukaryotes (4). *Saccharomyces cerevisiae* Mediator (*Sc*Mediator) has structurally distinct modules, referred to as tail, middle, head, and Cdk8 (3). *S. cerevisiae* tail module subunits *Sc*Med2, *Sc*Med3, and *Sc*Med15 stabilize each other's presence in the complex, facilitate interactions between DNA-bound transcriptional activators and the complex at highly induced promoters, and coordinate the activity of Mediator and other coactivators, such as the SAGA and Swi/Snf complex (3). Although it is known that *Sc*Med15 is a direct target for a variety of TADs (1), it is not well understood how these weak TAD-Mediator interactions mechanistically facilitate the action of the tail module and Mediator to stimulate high levels of transcription. One idea is that the interactions between DNA-bound TADs primarily serve to physically recruit Mediator to certain promoters. Nonclassical activators, in contrast to classical activators, are artificial constructs covalently linking a DNA binding domain (DBD) to a component of the transcription machinery, such as a Mediator subunit, that have been used to test the recruitment hypothesis (5). The ability of nonclassical activators, such as DBD fusions to Mediator subunits (i.e., Med2 and Med3), to affect upregulation of transcription has been taken as the central evidence that physical recruitment of Mediator to a specific promoter by the DBD is sufficient for activation of a reporter gene, and that classical activators work through a similar mechanism (6–8). A second important question surrounding the fungal Mediator subunit Med2 involves its distant orthologs (9), referred to as Tlo proteins, in the human fungal pathogens *Candida albicans* and *Candida dubliniensis*.

The *TLO* (for telomere localized) genes in *C. albicans* are uniquely encoded by 14 highly identical paralogs versus only 2 Tlo paralogs in the highly related but far less virulent fungal pathogen *C. dubliniensis*. In all other sequenced fungi there is only one Med2/Tlo ortholog and no clear orthologs in metazoan cells. Because the amplification of the *C. albicans TLO* genes is the most striking difference from the highly syntenic *C. dubliniensis* genome, it has been postulated that it is an important factor in the virulence of *C. albicans* (10, 11). Our recent finding that this amplification leads to a large population of free Tlo protein (in addition to the Mediator-associated form) in *C. albicans* (9) but not in *C. dubliniensis* (12) has led us to investigate the functional properties that the Tlo/Med2 protein alone may possess that would allow it to influence virulence.

Here, we report the presence of potent classical TADs in *C. albicans* α and β clade Tlo (*Ca*Tlo), the *C. dubliniensis* Tlo (*Cd*Tlo), and *S. cerevisiae* Med2 and Med3 proteins that are functionally and physically separable from their incorporation into the multisubunit complex. This finding suggests an alternative interpretation of the DBD-Mediator subunit fusion experiments and

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leads us to conclude that the direct recruitment of Mediator to a promoter is not sufficient for activated transcription. Our discovery of Mediator-associated TADs also has important implications for understanding how Mediator directs the activity of other coactivators (13, 14) and how the large excess of free Tlo protein in *C. albicans* (9, 15) might affect virulence gene expression.

MATERIALS AND METHODS

Plasmid construction. The complete list of plasmids and primers used in this study are available upon request. The details of the construction of these plasmids is presented here. For the overexpression of the *C. albicans* Tloα12ΔC (*Ca*Tloα12ΔC) and Med3-6His protein in *Escherichia coli* cells, we modified the bicistronic plasmid p*ET21b-TLOα12-MED3-6HIS* (9) to create p*ET21b-TLOα12ΔC-MED3-6HIS* by amplifying the truncated *TLOα12* using the primers LM032 and LM033, cleaving with BglII and EcoRI, and cloning into a BglII/EcoRI-digested p*ET21b-TLOα12-MED3-6HIS* backbone.

For expression of *Sc*Med2 and *Sc*Med3 fragments in *S. cerevisiae*, we used a single-copy plasmid with the *MED2* promoter followed by the coding region for glutathione *S*-transferase–hemagglutinin (GST-HA) fused to the N terminus of the Mediator fragment. The GST coding sequence from p*GEX4T-1* was amplified by ZL212/ZL213 and inserted into p*GADT7* (Clontech) to replace *GAL4AD* between the KpnI and BglII sites to create p*ADGST*. A fragment that contained the *ADH1* promoter and coding sequence for GST followed by an HA tag was excised from p*ADGST* by NaeI/NdeI digestion and cloned into p*415-BD*. In the resulting vector, the *ADH1* promoter was replaced by the *ScMED2* promoter, amplified by ZL220/ZL221, to generate p*MEDGST*. p*MEDGST* was digested by NaeI and NdeI, and the P_{ScMED2}-*GST-HA*-containing fragment was inserted into p*CUP1-BD-ScMED2*, *-ScMED2ΔN*, and *-ScMED2ΔC* between the NaeI and NdeI sites to generate the corresponding p*MEDGST* plasmids. p*MEDGST-ScMED3*, *-ScMED3ΔN*, and *-ScMED3ΔC* were constructed by subcloning individual coding sequence-containing fragments from the corresponding p*CUP1-BD* plasmid into p*MEDGST* through NdeI and PvuI digestion.

For the one-hybrid assays in *S. cerevisiae*, we created a single-copy plasmid in which the expression of the Gal4 DBD fusion protein to the TAD candidate was driven by the *S. cerevisiae CUP1* promoter in order to mitigate the toxicity of strong TADs. The *CUP1* promoter was chosen since previous work had indicated that its activation was independent of several important Mediator subunits (16). Our own work confirmed that the levels of induction of a pCUP1-LacZ construct are equal within experimental error for the mutant strains we used (data not shown). All CUG codons in the *C. albicans* and *C. dubliniensis* genes fused to the Gal4 DBD were changed to a universal serine codon. To construct plasmids for expression of Gal4 DBD fusion proteins in *S. cerevisiae*, the following procedure was followed. p*GBKT7* (Clontech) was digested by EcoRV and PvuII, and the 2,777-bp fragment was inserted into pRS314 cut at these sites. The region between the two PvuI sites on this intermediate plasmid (p*314-BD*), which contains the *TRP1* marker, was replaced by its counterpart from p*RS415*, which contains the *LEU2* marker, to generate *p415-BD*. The *ScCUP1* promoter (17) was amplified by ZL121/ZL120 from BY4742 genomic DNA, fused to part of the Gal4 DBD coding sequence (amplified by ZL122/ZL123 from p*GBKT7*) by fusion PCR, and inserted into p*415-BD* between the NaeI and XhoI sites to generate p*CUP1-BD*. To create p*CUP1-BD-ScMED2*, *-ScMED2ΔN*, and *-ScMED2ΔC*, the coding sequences for full-length *Sc*Med2 (amino acids [aa] 1 to 431 amplified by ZL214/ZL199), *Sc*Med2 C terminus (aa 156 to 431, amplified by ZL198/ZL199), and *Sc*Med2 N terminus (aa 1 to 160, amplified by ZL214/ZL219) were cloned into p*CUP1-BD* between the BamHI and NotI sites. The coding sequence for full-length *Sc*Med3 (aa 1 to 397, amplified by ZL245/ZL246) was inserted into p*CUP1-BD* between the XmaI and SalI sites to generate p*CUP1-BD-ScMED3*. Digestion of p*CUP1-BD-ScMED3* with NotI resulted in 2 fragments; the larger piece, which contained the vector backbone and *Sc*Med3 N terminus (aa 1 to 147) coding sequence, was self-ligated to generate p*CUP1-BD-ScMED3ΔC*, and the smaller piece, which contained the *Sc*Med3 C-terminal (aa 145 to 397) coding sequence, was subcloned into p*CUP1-BD* through the NotI site in the correct orientation to generate p*CUP1-BD-ScMED3ΔN*. The coding sequence for full-length *Ca*Tloα12 (aa 1 to 252) was obtained by digesting p*ET21b-TLOα12-MED3-6HIS* by NdeI and EcoRI. The coding sequence for the *Ca*Tloα12 N terminus (aa 1 to 166) was obtained by digesting p*ET21b-TLOα12ΔC-MED3-6HIS* (9) by NdeI and EcoRI. The coding sequence for the *Ca*Tloα12 C terminus (aa 167 to 252) was amplified from p*ET21b-TLOα12-MED3-6HIS* by ZL112/LM35 and digested by NcoI and EcoRI. Each of these fragments first was cloned into p*GBKT7* and then subcloned into p*CUP1-BD* between the XhoI and NotI sites to generate p*CUP1-BD-CaTLOα12*, *-CaTLOα12ΔC*, and *-CaTLOα12ΔN*. ZL114/ZL115 were used to amplify the coding DNA for the *Ca*Tloγ5 C terminus (aa 120 to 176, identical to the C termini of *Ca*Tloγ7, *Ca*Tloγ11, and unspliced gene product of *CaTLOγ13*) from *C. albicans* genomic DNA, and the PCR product was inserted into p*415-BD* at the BamHI and NotI sites and subcloned into the pCUP1-BD XhoI and NotI sites to generate p*CUP1-BD-CaTLOγ5ΔN*. DNA encoding aa 172 to 273 of *Ca*Tloβ2 was amplified by ZL116/ZL119 from *C. albicans* genomic DNA and fused with the fragment amplified by ZL117/ZL118 from p*CUP1-BD-TLOα12ΔN*, which encodes the remaining 14 amino acid residues of the Tloβ2 C terminus (FDNFDDFIGFDIND; conserved in *Ca*Tloα12) in frame with *GAL4BD* by fusion PCR. The final product, which includes the coding sequence for the entire *Ca*Tloβ2 C terminus (aa 158 to 273), first was cloned into p*415-BD* through the XhoI and NotI sites to generate p*415-BD-CaTLOβ2ΔN*. To change the CUG codon, which encodes *Ca*Tloβ2 Ser239 in *C. albicans*, to a common serine codon (UCG), two overlapping fragments containing the modified codon were amplified from p*415-BD-CaTLOβ2ΔN* by ZL117/ZL148 and ZL147/BDR, respectively, and sealed by fusion PCR. This final PCR product was cloned into p*CUP1*-BD between the XhoI and NotI sites to generate p*CUP1-BD-CaTLOβ2ΔN*. To construct p*CUP1-BD-CdTLO1ΔN* and p*CUP1-BD-CdTLO2ΔN*, ZL131/ZL132 and ZL192/ZL193 were used to amplify and clone *Cd*Tlo1ΔN (aa 199 to 320) and *Cd*Tlo2ΔN (aa 250 to 355) coding sequences, respectively, from *C. dubliniensis* genomic DNA into the p*415-BD* vector. The CUG codons of *Cd*Tlo1 (Ser280) and *Cd*Tlo2 (Ser322) were changed to UCG by fusion PCR using ZL117/ZL146/ZL145/BDR and ZL117/ZL194/ZL195/BDR, respectively, as previously described for the *Ca*Tloβ2 Ser239 codon. Each codon-modified DNA fragment was cloned into p*CUP1*-BD vector between the XhoI and NotI sites. The WT VP16 activation domain and the F442A mutant were amplified, with part of the GAL4 DBD, by ZL117/ZL133 from pSB202 and its F442A derivative (18) and subcloned into p*CUP1*-BD by XhoI and NotI. The full-length *Ca*Med3 (aa 1 to 197), *Ca*Med3 C terminus (aa 107 to 197), and *Ca*Med3 N terminus (aa 1 to 106) coding sequences were amplified from p*ET21b-TLOα12-MED3-6HIS* (9) by ZL163/ZL256, ZL257/ZL256, and ZL163/ZL281, respectively, and cloned into the BamHI and PstI sites of p*CUP1-BD* to generate p*CUP1-BD-CaMED3*, *-CaMED3ΔN*, and *-CaMED3ΔC*. To fuse different regions of the ScMed2 C terminus to the Gal4 DBD, DNA fragments amplified by ZL198/ZL261, ZL260/ZL199, ZL198/ZL269 ZL260/ZL261, and ZL270/ZL199, which encoded aa 156 to 385, 259 to 431, 156 to 268, 259 to 385, and 380 to 431, respectively, of ScMed2 from p*CUP1-BD-ScMED2* were individually cloned into the p*CUP1-BD* vector between the BamHI and NotI sites. DNA products amplified by ZL198/ZL262 and ZL263/ZL199 from p*CUP1-BD-ScMED2* were sealed by fusion PCR and cloned into the p*CUP1-BD* vector to generate *CUP1-BD-ScMED2-A/B* for the expression of the Gal4BD-fused *Sc*Med2 C terminus (aa 156 to 258 and 380 to 431), which lacks the N-rich domain. Coding DNA for ScMed3 C-terminal fragments aa 204 to 397, 204 to 346, and 145 to 203 was amplified by ZL268/ZL246, ZL268/ZL296, and ZL266/ZL295, respectively, from p*CUP1-BD-ScMED3* and cloned into the p*CUP1*-BD vector. p*CUP1-BD-ScMED3-aa282-397* was the self-ligation product of NcoI-digested p*CUP1-BD-ScMED3*. ZL297/ZL298 were used to amplify the

TABLE 1 List of *S. cerevisiae* strains used in this study

Strain	Genotype	Remarks	Reference
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	WT	22
13701	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 med2Δ::KAN^r*	Δ*med2*	22
14393	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 med3Δ::KAN^r*	Δ*med3*	22
yLM148	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 MED2::MED2ΔC-KAN^r*	*MED2ΔC*	This study
yLM149	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* MED3::*MED3ΔC-HIS3*	*MED3ΔC*	This study
yLM150	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 MED2::MED2ΔC-KAN^r MED3::MED3ΔC-HIS3*	*MED2ΔC MED3ΔC*	This study
yLM53	*med2Δ::TRP1 MED18::MED18-3FLAG-NAT^r*	Δ*med2 MED18-3FLAG*	This study; derived from references 23 and 24
yLM151	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 med3Δ::KAN^r MED18::MED18-3FLAG-NAT^r*	Δ*med3 MED18-3FLAG*	This study
yLM152	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 med2Δ::KAN^r MED3::MED3ΔC-HIS3 MED18::MED18-3FLAG-NAT^r*	Δ*med2 MED3ΔC MED18-3FLAG*	This study
yLM153	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 med2Δ::HIS3 med3Δ::KAN^r*	Δ*med2*Δ*med3*	This study
yLM246	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 MED7::MED7-3HA-HPH*	WT *MED7-3HA*	This study
yLM247	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 MED17::MED17-3HA-HPH*	WT *MED17-3HA*	This study
yLM248	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 MED2::MED2ΔC-KAN^r MED3::MED3ΔC-HIS3 MED7::MED7-3HA-HPH*	*MED2ΔC MED3ΔC MED7-3HA*	This study
yLM249	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 MED2::MED2ΔC-KAN^r MED3::MED3ΔC-HIS3 MED17::MED17-3HA-HPH*	*MED2ΔC MED3ΔC MED17-3HA*	This study

genomic region that contains the sequence encoding the aa 375 to 397 fragment of ScMed3.

The integrating plasmids used to create the *C. albicans* one-hybrid assay strains were constructed as follows. We replaced the *CaACT1* promoter in the integrating plasmid CIp-LexA (19), used to express the LexA DBD-TAD fusion proteins in the *C. albicans* one-hybrid assay strains, by the *CaMAL2* promoter. The regulatable *CaMAL2* promoter was used to mitigate the toxicity of some constructs in the *C. albicans* reporter strain cRC106 (19). Our earlier work has shown that the *CaMAL2* promoter functions independently of deletions in the Mediator tail module in *C. albicans* (20). Specifically, the *CaACT1* promoter in CIp-LexA (19) was replaced by the *CaMAL2* promoter by amplifying the *CaMAL2* promoter from pSFS2 (21) using ZL179/ZL180. This fragment was fused to the LexA DBD coding sequence, which was amplified by ZL181/ZL182 from *CIp-LexA* by fusion PCR. This p*MAL2-LexA* fragment was cloned into *CIp-LexA* between the XhoI and MluI sites to generate the p*MAL2-LexA* vector. Various *C. albicans* and *C. dubliniensis* Tlo and Med3 fragments were inserted into p*MAL2-LexA* between the MluI and PstI sites. Fragments containing *CaTLOα12*, *CaTLOα12ΔN*, and *CaTLOα12ΔC* were amplified by ZL168/ZL171, ZL169/ZL171, and ZL168/ZL189, respectively, from p*BSKS-TLOα12* (9). *TLOγ5ΔN* was amplified by ZL170/ZL172 from p*CUP1-BD-CaTLOγ5ΔN*, *CdTLO1ΔN* was amplified by ZL227/ZL228 from *C. dubliniensis* genomic DNA, *CaMED3ΔN* was amplified by ZL255/ZL256 from *C. albicans* genomic DNA, and the *CaGCN4* coding sequence was excised from *CIp-LexA-GCN4* (19) by MluI and PstI. All of these DNA fragments were subcloned into p*MAL2-LexA*. The *CaGCN4* coding sequence also was amplified by ZL229/ZL230 and subcloned into p*CUP1-BD* between the BamHI and NotI sites for the reporter assays in *S. cerevisiae*.

Vectors possessing DNA cassettes for integrating full-length *CdTLO1*, *CdTLO1ΔC*, and *SAT1* marker (as the vector control) into the *C. dubliniensis tloΔΔ* strain at the original *TLO1* locus were generated by inserting a *TLO1* downstream region amplified by ZL286/ZL287 into *pFA6a-3HA-SAT1* (9) between the Pme1 and SacII sites and then inserting the *Cd*TLO1 open reading frame (ORF) with its upstream sequence (amplified by ZL282/ZL299), *CdTLO1ΔC* (encoding aa 1 to 200) with the upstream sequence (amplified by ZL282/ZL300), or the upstream region itself (amplified by ZL282/ZL283) between the HindIII and AscI/BamHI sites. Similarly, the same *CdTLO1* downstream region and *CdTLO1*/*CdTLO1ΔC* coding region with the upstream sequence (amplified by ZL282/ZL284 or ZL282/ZL285) were sequentially cloned into *pFA6a-6HIS3FLAG-SAT1* for expressing the C-terminal 6His-3FLAG-tagged *Cd*Tlo1 or *Cd*Tlo1Δ*C* in the *tloΔΔ* strain.

Strain construction. The complete set of *S. cerevisiae* strains used in this study is listed Table 1 and the complete set of *Candida* species strains is listed in Table 2. The complete list of plasmids and primers used to construct these strains is available upon request. For purification of *C. albicans* Mediator containing Tloα12 or Tloα12ΔC, we used *C. albicans* strains (*TLOα12-6HIS-3FLAG* and *TLOα12ΔC-6HIS-3FLAG*) with the *6HIS-3FLAG* tag (9) integrated at the 3′ end of the full-length or truncated *TLOα12* (encoding aa 1 to 166) at the single chromosomal locus for *TLOα12*. A second set of *C. albicans* strains with an HA tag (9) integrated at the 3′ end of the full-length or truncated *TLOα12* (encoding aa 1 to 166) and a *6HIS-3FLAG* tag (9) integrated at the 3′ end of *MED8* was used to purify Mediator via its head module. Specifically, C-terminal tagging of *Ca*Med8 and full-length *Ca*Tloα12 in *C. albicans* was performed as described previously (9) in the BWP17 strain background (25). The primer pair ZL113/KPP037 was used to amplify the *TLOα12ΔC-3HA* and *TLOα12ΔC-6HIS-3FLAG* tagging cassettes from the p*FA-3HA-ARG4* plasmid (27) and p*FA-6HIS-FLAG-ARG4* plasmid (generated by replacing the *SAT1* marker in p*FA-6HIS-FLAG-SAT1* [9] with the *ARG4* marker), respectively. These cassettes were integrated into a single chromosomal copy and validated as previously described (9).

To create the *S. cerevisiae MED2* or *MED3* C-terminal truncation strains, BY4742 (Yeast Deletion Project [22]) was transformed by the PCR products amplified by ZL217/ZL062 from pFA6a-KanMX6 (28) or by ZL248/ZL250 from p*FA6a-HIS3MX6* (28) to generate yLM148 (*MED2ΔC*) or yLM149 (*MED3ΔC*), respectively. The latter DNA product also was used to replace the coding sequence of the Med3 C terminus with a *HIS3* marker in yLM148 to generate yLM150 (*MED2ΔC MED3ΔC*) and in 13701 (Δ*med2* mutant; Yeast Deletion Project [22]) to generate an intermediate strain. This intermediate Δ*med2 MED3ΔC* strain was triple *FLAG* tagged on the 3′ end of *MED18* as described previously (29) to generate yLM152. The procedure was used to triple *FLAG* tag the 3′ end of *MED18* in 14393 (Δ*med3*; Yeast Deletion Project [22]) to generate yLM151. To generate yLM153 (Δ*med2* Δ*med3*), the *MED2* ORF was replaced with a *KAN^r* marker in 14393 using a PCR product amplified by ZL218/ZL062 from p*FA6a-KanMX6*. yLM53 (Δ*med2/MED18-3FLAG*) was sporulated from a diploid strain resulting from mating MG107 (Δ*med2* [23]) and SHY349 (*MED18-3FLAG* [24]) and verified by the inability to utilize galactose, which confirms the absence of *MED2*, and immunoblotting to confirm the presence of FLAG-tagged *MED18*.

TABLE 2 List of *C. albicans* and *C. dubliniensis* strains used in this study

Strain	Genotype	Reference
BWP17	*MTL***a**/α *ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG arg4*Δ::*hisG*/*arg4*Δ::*hisG*	25
yLM154	*MTL***a**/α *ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG arg4*Δ::*hisG*/*arg4*Δ::*hisG MED8*::*MED8-6HIS-3FLAG-SAT1*/*MED8*::*MED8-6HIS-3FLAG-HIS1 TLOα12*::*TLOα12-3HA-ARG4*/-[a]	This study
yLM155	*MTL***a**/α *ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG arg4*Δ::*hisG*/*arg4*Δ::*hisG MED8*::*MED8-6HIS-3FLAG-SAT1*/*MED8*::*MED8-6HIS-3FLAG-HIS1 TLOα12*::*TLOα12*Δ*C-3HA-ARG4*/-	This study
cZL1	*MTL***a**/α *ura3*Δ::λ*imm*^{434}/*ura3*Δ::λimm^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG arg4*Δ::*hisG*/*arg4*Δ::*hisG TLOα12*::*TLOα12-6HIS-3FLAG-SAT1*/-	9
yLM156	*MTL***a**/α *ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG arg4*Δ::*hisG*/*arg4*Δ::*hisG TLOα12*::*TLOα12*Δ*C-6HIS-3FLAG-ARG4*/-	This study
cRC106	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *ade2*::*hisG*/*ade2*::*hisG*::[p*OPlacZ*]	19
yLM158	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *ade2*::*hisG*/*ade2*::*hisG*::[p*OPlacZ*] *RPS10*/*rps10*Δ::[p*MAL-LexA-URA3*]	This study
yLM159	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *ade2*::*hisG*/*ade2*::*hisG*::[p*OPlacZ*] *RPS10*/*rps10*Δ::[p*MAL-LexA-TLOα12-URA3*]	This study
yLM160	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *ade2*::*hisG*/*ade2*::*hisG*::[p*OPlacZ*] *RPS10*/*rps10*Δ::[p*MAL-LexA-TLOα12*Δ*N-URA3*]	This study
yLM161	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *ade2*::*hisG*/*ade2*::*hisG*::[p*OPlacZ*] *RPS10*/*rps10*Δ::[p*MAL-LexA-TLOα12*Δ*C-URA3*]	This study
yLM162	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *ade2*::*hisG*/*ade2*::*hisG*::[p*OPlacZ*] *RPS10*/*rps10*Δ::[p*MAL-LexA-CaMED3*Δ*N-URA3*]	This study
yLM163	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *ade2*::*hisG*/*ade2*::*hisG*::[p*OPlacZ*] *RPS10*/*rps10*Δ::[p*MAL-LexA-CdTLO1*Δ*N-URA3*]	This study
yLM164	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *ade2*::*hisG*/*ade2*::*hisG*::[p*OPlacZ*] *RPS10*/*rps10*Δ::[p*MAL-LexA-CaTLOγ5*Δ*N-URA3*]	This study
yLM165	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *ade2*::*hisG*/*ade2*::*hisG*::[p*OPlacZ*] *RPS10*/*rps10*Δ::[p*MAL-LexA-CaGCN4-URA3*]	This study
Wü284	*Candida dubliniensis* wild type	26
*tlo*ΔΔ	*tlo1*Δ::*FRT*/*tlo1*Δ::*FRT*; *tlo2*Δ::*FRT*[b]	12
yLM250	*tlo1*Δ::*FRT*/*tlo1*Δ::*SAT1*; *tlo2*Δ::*FRT*	This study
yLM251	*tlo1*Δ::*FRT*/*tlo1*Δ::P_{TLO1}-*TLO1-SAT1*; *tlo2*Δ::*FRT*	This study
yLM252	*tlo1*Δ::*FRT*/*tlo1*Δ::P_{TLO1}-*TLO1*Δ*C-SAT1*; *tlo2*Δ::*FRT*	This study
yLM253	*tlo1*Δ::*FRT*/*tlo1*Δ::P_{TLO1}-*TLO1-6HIS-3FLAG-SAT1*; *tlo2*Δ::*FRT*	This study
yLM254	*tlo1*Δ::*FRT*/*tlo1*Δ::P_{TLO1}-*TLO1*Δ*C-6HIS-3FLAG-SAT1; tlo2*Δ::*FRT*	This study

[a] In BWP17, one allele of *TLOα12* is missing due to a truncation event at the far right end of chromosome V. Therefore, BWP17, as well as its derivatives (yLM154, yLM155, cZL1, and yLM156), contains only a single copy of *TLOα12* or its modified form.
[b] There is only one allele of *TLO2* present in Wü284 and its derivatives due to a chromosomal truncation event.

To generate each strain (yLM158 to yLM165) for *C. albicans* one-hybrid assays, p*MAL2-LexA* or the corresponding derivative was linearized by StuI digestion before being transformed into cRC106 (19). Transformants were selected on SC-uridine plates, and correct integration was tested by PCR using ZL093/ZL174 and ZL173/ZL094.

To complement a *C. dubliniensis tlo*Δ/Δ strain (12) with nontagged or *6HIS-3FLAG*-tagged *CdTLO1*/*CdTLO1*Δ*C*, individual integrative DNA cassettes were released from the corresponding vector by HindIII and SacII digestion and transformed into *tlo*ΔΔ cells by electroporation. The correct integration was tested by ZL288/LM21 (for the 5′ junction) and KPP63/ZL289 (for the 3′ junction) and Western blotting (anti-FLAG), if applicable.

C-terminal tagging of Med7 and Med17 was done as described previously (29). Targeting DNA cassettes were amplified using pFA6a-3HA-HPH (30) as the template, as well as primers ZL007/ZL008 for *MED17* tagging and ZL043/ZL044 for *MED7*. Transformants were selected by hygromycin B, and the correct integration was confirmed by both PCR (KanB/ZL012 for *MED17* tagging and KanB/ZL045 for *MED7* tagging) and Western blotting (anti-HA).

Protein purification. yLM53 (Δ*med2*), yLM151 (Δ*med3*), or yLM152 (Δ*med2 MED3*Δ*C*) strains carrying the indicated p*MEDGST* plasmid were grown in SC-leucine liquid medium to late log phase and processed as described previously (29, 31) with modifications. Whole-cell extract (~200 mg) was applied to 200 μl anti-FLAG-M2 agarose (Sigma), washed, and on-column treated by Benzonase [final concentration at 500 U/ml in 25 mM HEPES KOH (pH 7.6), 10% glycerol, 0.01% NP-40, 300 mM potassium acetate (KOAc), 2.5 mM magnesium acetate {$Mg(OAc)_2$}] (EMD) at room temperature for 30 min. Mediator complex was eluted by 50 μg/ml 3FLAG peptide.

Purification of *Candida* sp. Mediator complex was performed as described previously (9). Expression, immobilized metal affinity chromatography (IMAC) purification, and size-exclusion chromatography analysis of recombinant *C. albicans* Tloα12ΔC/Med3-6His protein complex were performed as described previously (9).

Liquid β-gal assays. For *S. cerevisiae* β-galactosidase (β-gal) reporter assays, the BY4742 strain was cotransformed with reporter plasmid with multiple Gal4 binding sites, followed by the *CYC1* core promoter fused to LacZ (32) and a single-copy plasmid expressing a particular pCUP1-driven Gal4BD fusion protein. Specifically, the BY4742 strain was cotransformed with p*LGSD5* (32) and the indicated plasmid expressing a particular pCUP1-driven Gal4 DBD fusion protein. Transformants were selected on SC-uracil-leucine plates. To determine the activation potential of a given fusion protein, at least 10 independent colonies from at least two independent transformations first were grown overnight in 2 ml of specified SC-uracil-leucine liquid medium, which contained 6.7 g/liter yeast nitrogen base (YNB; without amino acids and with ammonium sulfate; low Fe, Zn, Mn, Cu; US Biological), 2 g/liter dropout mix (synthetic medium lacking adenine, histidine, leucine, tryptophan, and uracil, without YNB) (US Biological), 2% glucose, 30 mg/liter adenine, 20 mg/liter histidine, 20 mg/liter tryptophan, 200 μg/liter $FeCl_3$, 400 μg/liter $ZnSO_4 \cdot 7H_2O$, 303 μg/liter $MnSO_4 \cdot H_2O$, and 800 nM $CuSO_4$. Fresh overnight cultures were diluted 1:20 in 3 ml of the same medium and

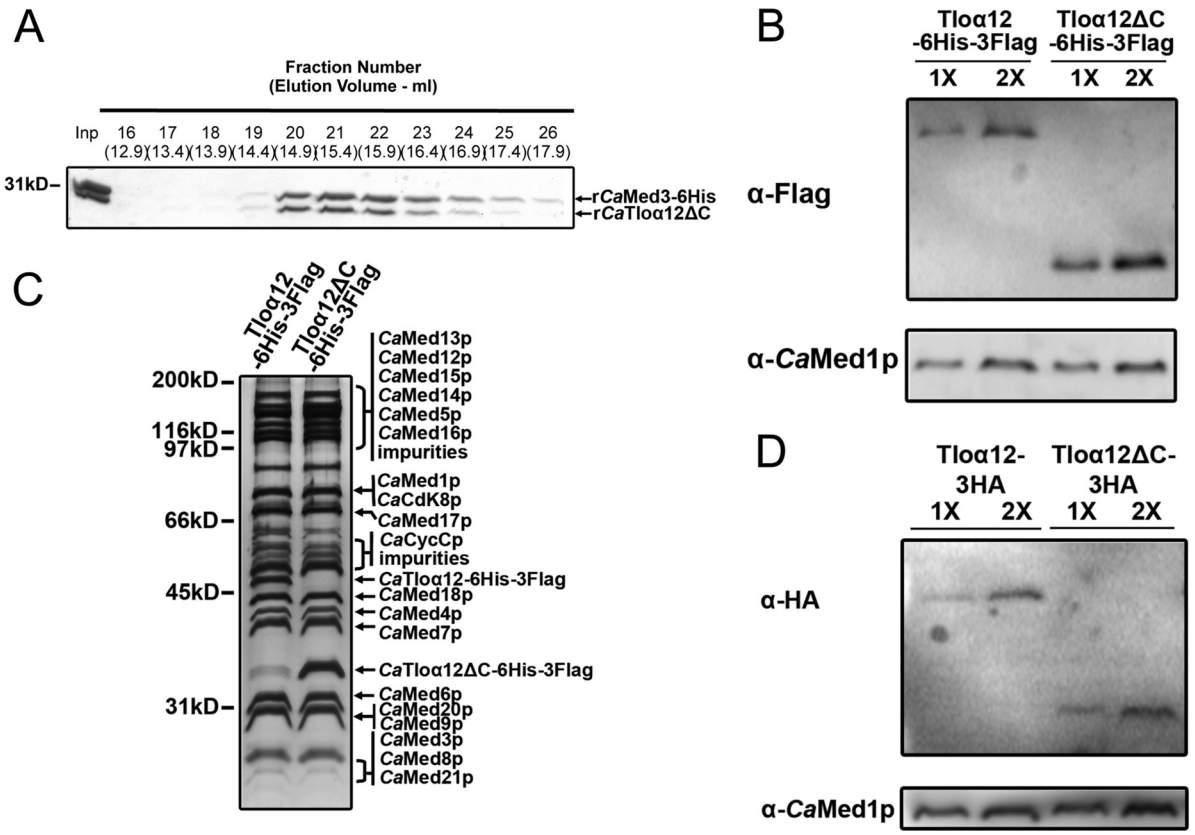

FIG 1 The N terminus of a *C. albicans* Tlo protein is necessary and sufficient for interactions with *Ca*Med3 and incorporation into *C. albicans* Mediator. (A) Coexpressed recombinant *Ca*Tloα12ΔC and *Ca*Med3-6His copurify by IMAC and size-exclusion chromatography. Lysates of *E. coli* cells coexpressing *C. albicans* Tloα12ΔC and Med3-6His protein were subjected to IMAC purification, and the eluates were further analyzed by size-exclusion chromatography (void volume, ~7 ml; Superose 6). Input (Inp; IMAC eluate) and the fractions, which came off the column at the indicated elution volumes, were resolved by SDS-PAGE (12.5%) and stained by Coomassie blue. (B and C) Affinity purification of Mediator from *C. albicans* strains with a tag placed on the C-terminal end of full-length Tloα12 [*TLOα12-6HIS-3FLAG* (cZL1)] or Tloα12Δ*C* [*TLOα12ΔC-6HIS-3FLAG*(yLM156)] results in isolation of an intact complex as monitored by an immunoblot demonstrating equal *Ca*Med1 content (B) and by silver stain of the isolated complexes resolved by 10% SDS-PAGE (C). (D) Immunoblot showing that a similar level of Tloα12-3HA and Tloα12ΔC-3HA protein are present in *Ca*Mediator purified from the strains yLM154 and yLM155, respectively, using a tag on the head module subunit Med8 (Med8-6His-3FLAG). Comparable amounts of anti-FLAG–agarose eluate (calibrated by FLAG signal; not shown) were resolved by SDS-PAGE and probed by anti-HA and anti-*Ca*Med1 antibody.

grown for 2 to 3 doublings. β-Galactosidase activity was measured by the SDS-chloroform method as described previously (23). Normally, reaction mixtures were incubated in a 30°C water bath with shaking for 15 to 40 min to reach a final A_{420} reading in the range of 0.1 to 0.7. The optical density at 600 nm (OD_{600}) and A_{420} were determined by a Beckman Coulter DU-7300 spectrophotometer.

To induce the activator fusion proteins in the *C. albicans* one-hybrid experiments, the cells were grown in 2% maltose leading up to the measurement of β-gal activity. In *C. albicans* one-hybrid experiments, the β-galactosidase activity of each strain was based on measurements of at least 5 independent PCR-verified transformants. Cells first were grown in 2 ml SC plus maltose (6.7 g/liter YNB [Difco], 2 g/liter synthetic dropout mix lacking uracil [US Biological], 200 μM uridine, and 2% maltose) overnight and diluted ~1 to 20 in 3 ml fresh SC plus maltose. After 2 to 3 doublings, β-galactosidase activity was measured by the SDS-chloroform method (23). The reaction time could be as long as 90 to 120 min to detect weak β-gal activities typically associated with this assay in *C. albicans* (19). Miller units for the reactions were calculated by the following formula: $1{,}000 \times A_{420}/(T \times V \times OD_{600})$, where A_{420} is the absorbance of the reaction product at 420 nm, T is the reaction time in minutes, OD_{600} is the optical density at 600 nm of the cell resuspension used for the assay, and V is the volume of the cell resuspension used for the assay in milliliters.

RT-qPCR. To test *GAL* gene induction in *S. cerevisiae*, a given strain first was grown in SC plus raffinose (2%) overnight and diluted into fresh SC plus raffinose. After 2 to 3 doublings, cells were collected and resuspended in SC plus galactose (2%). At 0 min, 20 min, or 90 min after transfer to SC plus galactose, cultures were aliquoted and processed for RNA preparation and reverse transcription-quantitative PCR (RT-qPCR) as described previously (9). qPCR was performed and analyzed by the relative standard curve method (Applied Biosystems). Specifically, the relative standard curve of *SCR1* was generated by series dilution of the cDNA sample prepared from the WT cells at 0 min and used to determine the relative abundance of *SCR1* in all of the samples (1 to 100 diluted). The relative standard curves for *GAL1* and *GAL10* were generated by series dilution of the cDNA samples prepared from WT cells after 90 min of galactose induction. Primers ZL241/ZL242, ZL243/ZL244, and ZL275/ZL276 were used to quantify the relative mRNA abundance of *GAL1*, *GAL10*, and *SCR1*, respectively.

GAL gene induction in *C. dubliniensis* was performed using an approach similar to that for *S. cerevisiae*. Cells were grown in SC plus glucose/uridine instead of SC plus raffinose/uracil due to the incapability for *C. dubliniensis* utilizing raffinose or uracil. The abundance of *CdGAL1* and *CdGAL10* transcripts before and after induction (30 min in SC plus galactose/uridine) were determined by RT-qPCR (primers ZL382/ZL383 for *CdGAL1* and primers ZL384/ZL385 for *CdGAL10*) with *CdACT1* as the reference.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) experiments were performed as described previously (33), with

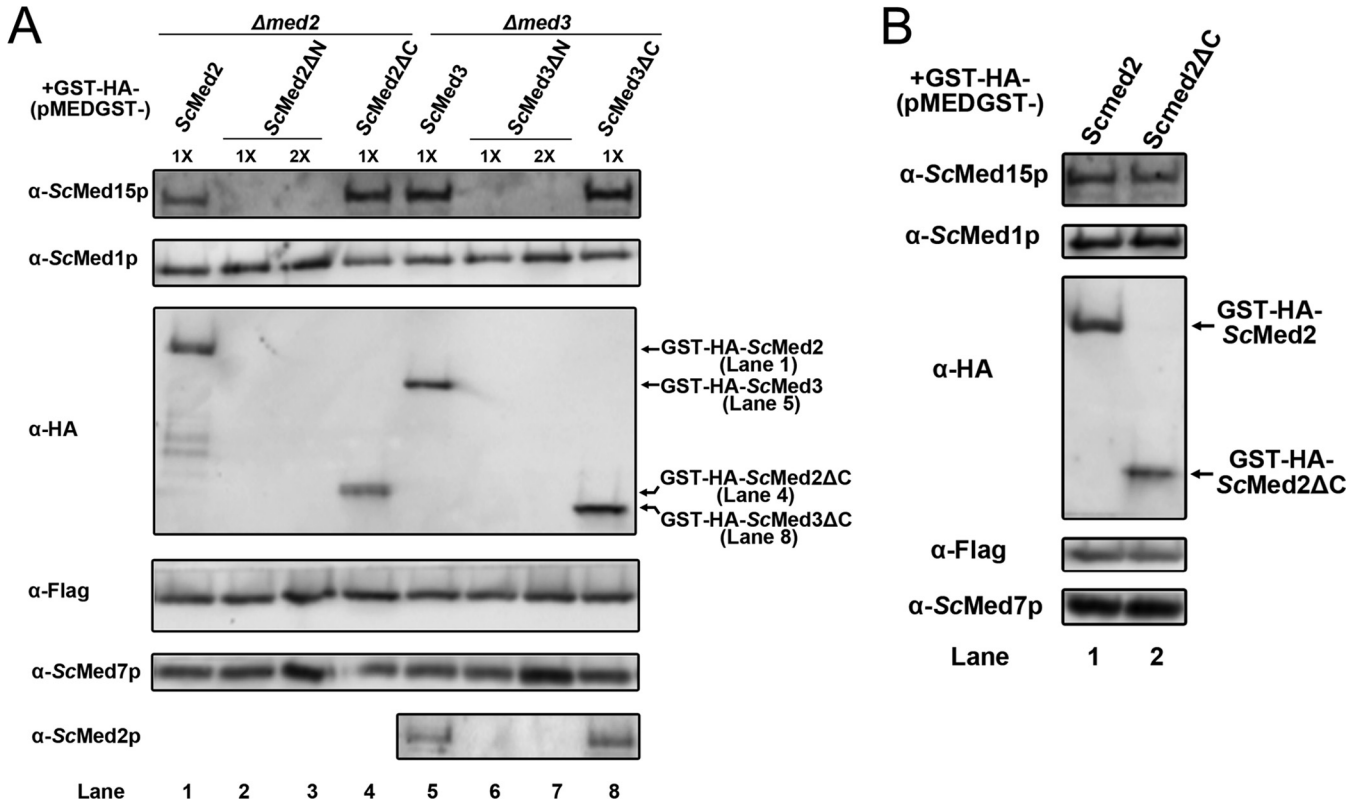

FIG 2 Ability of N- and C-terminal fragments of *S. cerevisiae* Med2 and Med3 to associate with Mediator. (A) The N termini of GST-Med2 and GST-Med3 fusion proteins are necessary and sufficient for association with *Sc*Mediator complex purified from Δ*med2 MED18-3FLAG* (yLM53) and Δ*med3 MED18-3FLAG* (yLM151) strains, respectively. Shown is an immunoblot comparing the composition of Mediator complex affinity purified from the Δ*med2 MED18-3FLAG* (yLM53) strain carrying p*MEDGST-ScMED2-HA* (lane1), p*MEDGST-ScMED2*Δ*N-HA* (lanes 2 and 3), and p*MEDGST-ScMED2*Δ*C-HA* (lane 4) and from the Δ*med3 MED18-3FLAG* (yLM151) strain carrying p*MEDGST-ScMED3* (lane 5), p*MEDGST-ScMED3*Δ*N-HA* (lanes 6 and 7), and p*MEDGST-ScMED3*Δ*C-HA* (lane 8) resolved by SDS-PAGE (10%) and probed by the indicated antibodies. The inability of the GST-ScMed2ΔN and GST-ScMed3ΔN proteins to associate with the complex was not a result of reduced expression levels of the truncations (data available upon request). (B) The tail module of *Sc*Mediator can be stably assembled and associated with the complex in the absence of the C termini of *Sc*Med2 and *Sc*Med3. Shown is an immunoblot comparing the composition of the *Sc*Mediator complex purified from a Δ*med2 MED3*Δ*C MED18-3FLAG* strain (yLM152) expressing GST-HA-ScMed2 (lane 1) and GST-HA-ScMed2ΔC (lane 2) from the corresponding p*MEDGST* plasmid.

modifications. yLM246, yLM247, yLM248, and yLM249 were grown overnight in SC plus raffinose, diluted in fresh SC plus raffinose, and allowed to experience 2 or 3 cell divisions before the culture was collected, washed, and continued to grow in SC plus galactose for 90 min. After 20 min of cross-linking in 1% formaldehyde, the cells were lysed by five 20-s bead beatings (Biospec). Crude chromatin samples first were probe sonicated (Fisher) at 30% amplitude for three 8-s sonications and further sheared by a Biodistruptor (high settings; 5 min four times; 30 s on/30 s off). Cross-linked Mediator-chromatin complexes were immunoprecipitated by F-7 HA antibody (Santa Cruz) and Dynabeads protein G (Life Technologies). After reverse cross-linking, DNA was recovered by a PCR purification kit (Qiagen). The recruitment pattern of Mediator along the *ScGAL1/10* locus was mapped by real-time PCR using primer pairs of ZL436/ZL437, G2-F/G2-R, ZL410/ZL411, G4-F/G4-R, and ZL241/ZL242. The enrichment of each DNA fragment was calculated by the percentage of input recovered in the ChIP product and compared between strains after being normalized to the recovery (percent input) at *PMA1* promoter (by primers ZL462/ZL463). *PMA1* is an actively transcribed gene whose expression level is *MED2*/*MED3* independent (34) and whose promoter has a high constitutive occupancy of Mediator (35).

Growth assays. The indicated *S. cerevisiae* or *C. dubliniensis* strains were grown overnight in YPD. After being washed in water, cells first were diluted to 3×10^6 cells/ml, and diluted 1:10 into 3×10^2 cells/ml, and spotted on YPD, YP plus 2% galactose plus 1 μg/ml antimycin A (AA), or YPD plus 5 mM H_2O_2 plates. Plates were incubated at 30°C. Liquid and agar media for growing *C. dubliniensis* were supplemented with 0.1 mM uridine.

Immunoblotting. Immunoblotting was performed as described previously (9, 29).

RESULTS

The N-terminal domains of *S. cerevisiae* Med3, *S. cerevisiae* Med2, and *C. albicans* Tlo are necessary and sufficient for the incorporation of these Mediator subunits into the complex. In this work, we show that the C-terminal domains of the *C. albicans* α and β clade (15) Tlo proteins, the *C. dubliniensis* Tlo proteins, and *S. cerevisiae* Med2 (*Sc*Med2) and Med3 (*Sc*Med3) can serve as potent transcriptional activation domains independently of their incorporation into the Mediator complex. Although TADs do not share any easily recognizable motifs or structures (36, 37), the C termini of the α and β clade Tlo proteins do have a pattern of acidic residues interspersed with hydrophobics that is characteristic of acidic TADs (38). This observation, plus the potent ability of full-length *Sc*Med2 and *Sc*Med3, compared to other Mediator subunits, to activate a reporter gene when fused to the LexA DBD (8) led us to hypothesize that the Tlo/Med2 and Med3 C termini

TABLE 3 Activation by Gal4-Med2/Tlo fusions in *S. cerevisiae*[a]

Gal4 DBD fusion protein (driven by p*CUP1*)	β-Gal reporter activity (Miller units, means ± SD[b])
Empty vector	<5
*Ca*Tloα12	507 ± 98
*Ca*Tloα12ΔN	968 ± 151
*Ca*Tloα12ΔC	<5
VP16	1,131 ± 229
VP16 F442A	229 ± 27
*Ca*Gcn4	941 ± 149
*Ca*Tloβ2ΔN	324 ± 53
*Ca*Tloγ5ΔN	<5
*Cd*Tlo1ΔN	1,109 ± 268
*Cd*Tlo2ΔN	1,431 ± 198
*Sc*Med2	63 ± 17
*Sc*Med2ΔN	642 ± 120
*Sc*Med2ΔC	<5
*Sc*Med3	736 ± 110
*Sc*Med3ΔN	747 ± 97
*Sc*Med3ΔC	32 ± 10
*Ca*Med3	38 ± 7
*Ca*Med3ΔN	<5
*Ca*Med3ΔC	44 ± 11

[a] Using an *S. cerevisiae* one-hybrid system, the Gal4 DNA binding domain (DBD) was fused to various *S. cerevisiae*, *C. albicans*, or *C. dubliniensis* Mediator gene fragments, and the activation of a *GAL4-CYC1-LACZ* reporter was monitored. The TAD activities of known classical activation domains (*Ca*Gcn4, VP16, and an attenuated VP16 mutant [F442A]) also were measured as controls.
[b] SD, standard deviations.

TABLE 4 Activation by LexA-Tlo fusions in *C. albicans*[a]

LexA fusion protein (driven by p*MAL2*)	β-Gal reporter activity (Miller units, means ± SD)
Empty vector	0.9 ± 0.1
*Ca*Tloα12	21.3 ± 7.9
*Ca*Tloα12ΔN	92.3 ± 12.5
*Ca*Tloα12ΔC	1.1 ± 0.3
*Ca*Gcn4	8.9 ± 1.6
*Ca*Tloγ5ΔN	0.7 ± 0.1
*Cd*Tlo1ΔN	155.1 ± 36.0
*Ca*Med3ΔN	1.1 ± 0.1

[a] Using the *C. albicans* one-hybrid system derived from the one developed by Russell and Brown (19), we fused the LexA DNA binding domain N terminal to various fragments of *C. albicans* and *C. dubliniensis* genes and monitored the activation of a *LEXA-ADH1-LACZ* reporter.

were classical activation domains. We also hypothesized that the N termini of these proteins were necessary and sufficient for the association of these subunits with Mediator, and that the C-terminal TAD activity was independent of incorporation into the complex.

We determined how the N and C termini of Med2 (Tlo) and Med3 contributed to the stability of the Mediator tail module within the entire complex using a protein purification approach. We divided the fungal Med2/Tlo and Med3 proteins, which form a heterodimer within fungal Mediator complexes (9, 39), into N- and C-terminal domains using a secondary-structure prediction algorithm (PSIPRED). This analysis suggested that the N termini consist of amphipathic α-helices, which most likely interact to form bundles with highly hydrophobic areas buried, and that the C termini are unstructured. Coexpression of the N terminus of recombinant *C. albicans* Tloα12, one of seven α-clade Tlo proteins that exist both as Mediator subunits and in a free form (9), and *C. albicans* Med3 (*Ca*Med3) in *E. coli* leads to the formation of a cocomplex that is stable over several purification steps (Fig. 1A). Consistent with the idea that the C termini of the Tlo proteins also are dispensable for their incorporation into Mediator, the C terminus of Tloα12 was not required for purification of intact Mediator from a *C. albicans* strain (*TLOα12ΔC-6HIS-3FLAG*) with an affinity tag on the truncated Tlo subunit (Fig. 1B and C) or from a *C. albicans* strain (*MED8-6HIS-3FLAG*) with an affinity purification tag on a head module subunit (Fig. 1D). The C termini of *Sc*Med2 and *Sc*Med3 are similarly dispensable for their incorporation into the *S. cerevisiae* complex. FLAG-agarose purification of Mediator from a *S. cerevisiae* Δ*med2* (or Δ*med3*) strain, which were FLAG tagged on the Med18 subunit and expressed the N or C termini of *Sc*Med2 (or *Sc*Med3) fused to GST, showed that only the N termini of these subunits were incorporated into an intact complex (Fig. 2A). Purification of an intact *Sc*Mediator from a FLAG-tagged Δ*med2 MED3ΔC* strain expressing GST-HA-*Sc*Med2Δ*C* further shows that the tail module of the complex can be stably assembled in the absence of the C termini of both ScMed2 and ScMed3 (Fig. 2B). Having demonstrated that the C termini of the Tlo proteins ScMed2 and ScMed3 were neither necessary nor sufficient for association with their respective Mediator complexes, we sought to formally demonstrate that these domains possessed potent TAD activity.

The C termini of *S. cerevisiae* Med3, *S. cerevisiae* Med2, *C. dubliniensis* Tlo, and *C. albicans* α and β clade Tlo proteins are potent transcriptional activation domains. We used one-hybrid systems in *S. cerevisiae* (with constructs modified from reference 18) and *C. albicans* (19) to measure the activation potential of various full-length, N-terminal, and C-terminal Med2(Tlo) and Med3 fragments. The DNA binding domain fusion proteins were under the control of an inducible promoter in both *S. cerevisiae* (*CUP1*) (17) and *C. albicans* (*MAL2*) (21) to mitigate the toxicity of potent activators and provide for comparable levels of fusion protein expression among the constructs. Fusion of the full length and C terminus of *C. albicans* Tloα12 to a heterologous DNA binding domain (Gal4 DBD) led to high levels of activation of a reporter in otherwise wild-type (WT) *S. cerevisiae* (Table 3) and *C. albicans* (Table 4) strains, while the Gal4 DBD-*C. albicans* Tloα12 N-terminal fusion did not. The signal for the C terminus was comparable to prototypical TADs in VP16 (36) and *C. albicans* Gcn4 (19) (Tables 3 and 4). Akin to classical activation domains, the *C. albicans* Tloα12 TAD is a potent activator in two distantly related fungi. TAD activity was conserved in the C terminus of the β clade *C. albicans* Tlo (Tloβ2) and the C termini of the Tlo proteins (*Cd*Tlo1 and *Cd*Tlo2) of the closely related human fungal pathogen *C. dubliniensis* but not in the C terminus of a γ clade *C. albicans* Tlo (Tables 3 and 4). This finding represents the first clear functional distinction between the highly expressed α and β clade Tlo proteins and the far more weakly expressed (15) γ clade Tlo proteins. Despite virtually no sequence similarity (and an additional ~200 amino acids), the C terminus of *Sc*Med2 is also a potent activator of transcription, while its Mediator-associated N terminus is not (Table 3). We also have found a TAD in the C terminus of *Sc*Med3 (Table 3), which contains a polyglutamine repeat. Polyglutamine repeats commonly overlap, and contribute to, TAD activity (40–42). The C terminus of *Ca*Med3, which does not have polyglutamine repeats, does not possess TAD activity

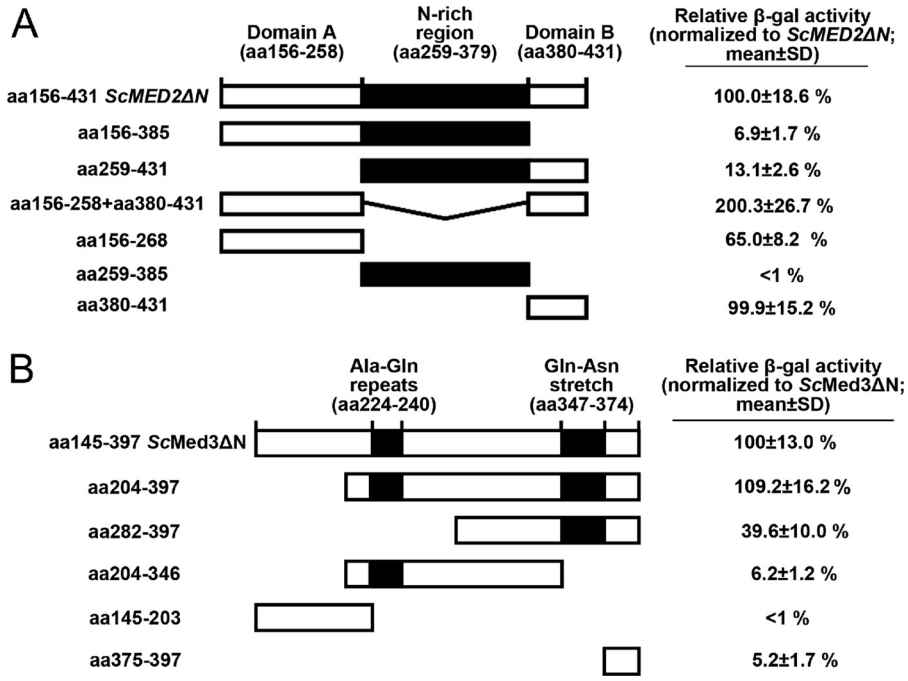

FIG 3 Subdomains of *S. cerevisiae* Med2 and Med3 C termini that contain TAD activity. (A) TAD activity in the ScMed2 C terminus resides within domain A (aa 156 to 258) and domain B (aa 380 to 431), while the asparagine (N)-rich region that separates them appears to have an attenuating effect on the TAD activity of domains A and B. β-Galactosidase activities of various Gal4DBD-Med2 constructs were measured as described for Table 1. (B) Full TAD activity in the ScMed3 C terminus requires both glutamine-rich domains, Ala-Gln (aa 224 to 240) and Gln-Asn (aa 347 to 374), to be present. β-Galactosidase activities of various Gal4DBD-Med3 constructs were measured as described for Table 3.

(Tables 3 and 4). While the polyglutamine repeats are required for the TAD activity of the *Sc*Med3 C terminus, the asparagine-rich region in the *Sc*Med2 C terminus actually appears to moderately attenuate its TAD activity rather than facilitate it (Fig. 3). Interestingly, the full-length Med2/Tlo fusions both were less active than their C-terminal-only counterparts, suggesting some form of autoinhibition. An increase in the TAD activity of full-length Med2 fusions in strains lacking WT Med2 suggests incorporation into Mediator may relieve this autoinhibition (Table 5). Interestingly, a good portion of this increase appears to be attributable to the presence of the *Sc*Med3 TAD (Table 5). We also found that the N terminus of *Sc*Med3 and the N-terminal and full-length *Ca*Med3 possessed some weak activation potential in *S. cerevisiae* (Table 3), despite the C terminus of *Ca*Med3 having no activation potential. This TAD activity appears to originate from the Med3 N termini recruiting non-Mediator-bound free Med2/Tlo and its associated TAD to the promoter, since the Med3 N-terminal TAD activity is entirely dependent on the presence of the Med2 C terminus (Table 5). Our findings and the published data (8) make it likely that Med2 and Med3 possess the only potent activation domains in *S. cerevisiae* Mediator, although previous work on *S. cerevisiae* (8) suggests there is a weak TAD in Med15. It also is possible that the DBD-Med15 fusion TAD activity observed in that previous work (8) resulted from the recruitment of a small amount of Med2/Med3/Med15 trimeric complex (43) that can activate the reporter. Either of these scenarios also suggests an alternative interpretation of earlier work showing that a mutation in *MED15*, which enables a direct interaction with the Gal4 DBD

TABLE 5 TAD activity of Med2 and Med3 in WT and mutant *S. cerevisiae* strains

Gal4 DBD fusion protein (driven by p*CUP1*)	β-Galactosidase reporter activity[a] (Miller units, means ± SD)				
	WT	Δ*med2*	Δ*med2 MED3*ΔC	Δ*med3*	Δ*med3 MED2*ΔC
*Sc*Med2	63 ± 17	665 ± 151	111 ± 29	<5	<5
*Sc*Med2ΔN	642 ± 120	404 ± 54	426 ± 82	395 ± 85	507 ± 66
*Sc*Med2ΔC	<5	94 ± 19	<5	<5	<5
*Sc*Med3	736 ± 110	273 ± 47	316 ± 30	830 ± 142	761 ± 85
*Sc*Med3ΔN	747 ± 97	291 ± 43	398 ± 52	358 ± 71	365 ± 36
*Sc*Med3ΔC	32 ± 10	<5	<5	336 ± 79	<5
*Ca*Med3	38 ± 7	<5	ND	687 ± 121	<10
*Ca*Med3ΔN	<5	<5	ND	<5	ND
*Ca*Med3ΔC	44 ± 11	<5	ND	655 ± 129	<10

[a] Using an *S. cerevisiae* one-hybrid system, the Gal4 DBD was fused to various *S. cerevisiae* Med2 or Med3 domains, and the activation of a *GAL4-CYC1-LACZ* reporter was measured in WT, Δ*med2*, Δ*med2 MED3*ΔC, Δ*med3*, and Δ*med3 MED2*ΔC strain backgrounds. ND, not determined.

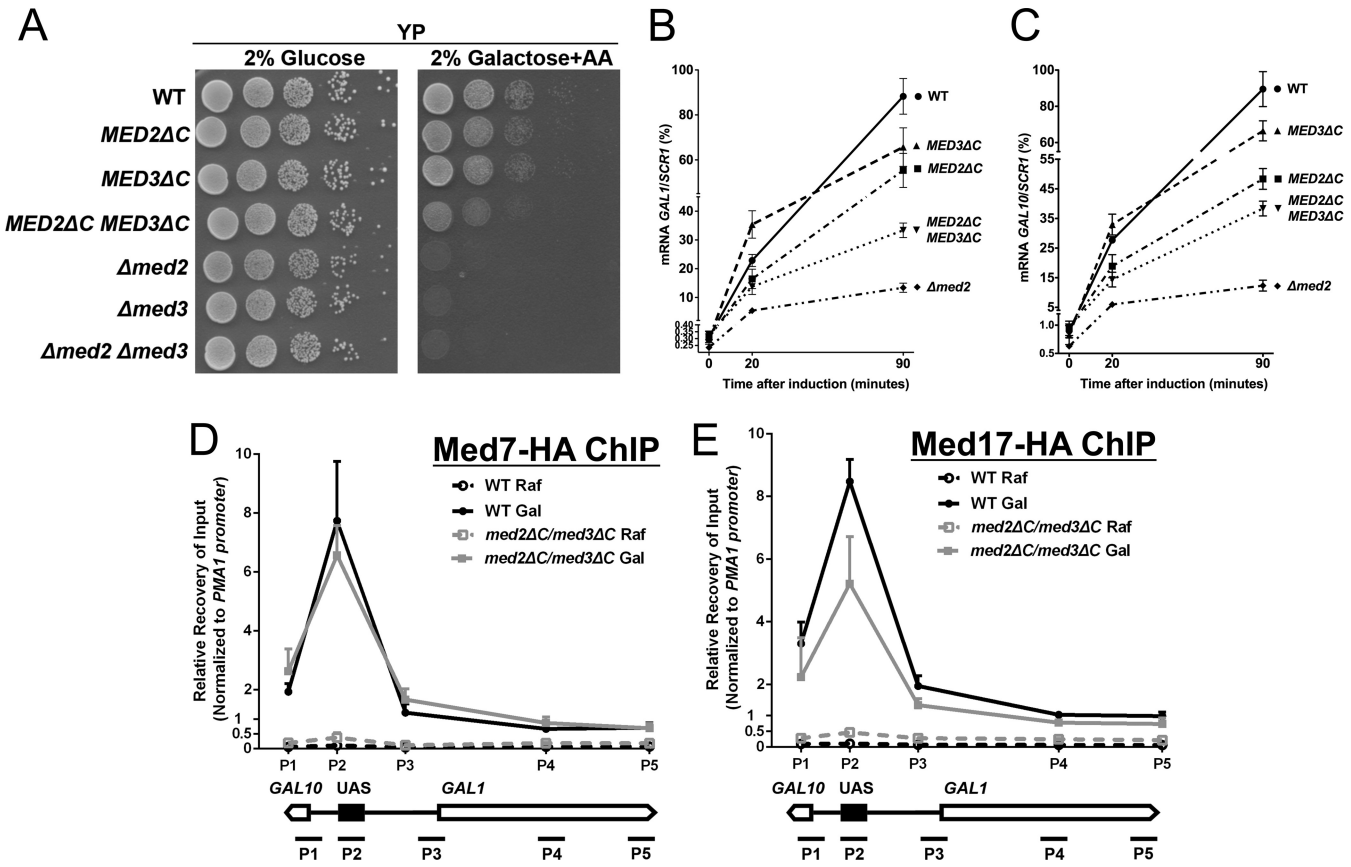

FIG 4 Deletion of the C-terminal TADs of *S. cerevisiae* Med2 and Med3 affect *GAL* gene expression. (A) Series dilution growth assay testing the fitness of the indicated *med2* and *med3* mutants on a YPD and YP galactose (supplemented with 2 μg/ml antimycin A [AA]) plate. An *S. cerevisiae* strain lacking the C-terminal TADs of both Med2 and Med3 (yLM150) has an intermediate growth defect using galactose as a carbon source compared to Δ*med2* (13701) (Yeast Deletion Project), Δ*med3*(14393), and Δ*med2*/Δ*med3* (yLM153) strains. (B and C) RT-qPCR comparing the activation of *GAL1* (B) and *GAL10* (C) in the WT (BY4742) and indicated mutant strains upon galactose induction. An *S. cerevisiae* strain lacking the C-terminal TADs of both Med2 and Med3 (yLM150) has an intermediate defect in induction of the *GAL1* (B) and *GAL10* (C) genes upon shifting the carbon source from raffinose to galactose compared to a Δ*med2*(13701) strain. Each data point (means ± standard deviations [SD]) represents, in total, five independent measurements from two independent biological replicates. The Δ*med2*, Δ*med3*, and Δ*med2* Δ*med3* mutations all have comparable effects on induction of *GAL1* and *GAL10* (data available upon request). (D and E) Anti-HA ChIP assays in wild-type and *med2*Δ*C*/*med3*Δ*C* strains with an HA tag on *MED7* (D) or *MED17* (E) show that the observed defect in *GAL* gene induction (B and C) does not result from an inability of Gal4 to recruit Mediator to the *GAL1*,*10* UAS upon shifting from raffinose (Raf) to galactose (Gal).

(no TAD), allowed the Gal4 DBD to activate a reporter gene without its TAD (44). This result originally was interpreted as supporting the idea that the direct recruitment of Mediator was sufficient for activated transcription (44). It is possible, however, that the Gal4 DBD was recruiting a Mediator tail module subcomplex that functioned as a classical TAD. The existence of potential TADs in the remaining subunits of the *C. dubliniensis* and *C. albicans* Mediator is still an open question. Our characterization of TADs conserved through divergent ascomycetes supports the idea that the activated transcription previously observed in full-length *Sc*Med2 and *Sc*Med3 fusions (8) is the result of a classical TAD rather than a nonclassical one.

The Mediator-associated activation domains of *S. cerevisiae* Med2 and Med3 are required for full induction of the *GAL1*,*10* promoter. Med2 and Med3, in combination with the activator target Med15, primarily positively regulate a subset of genes that are highly induced in response to environmental stimuli in *S. cerevisiae* (2). A function of the TADs associated with these subunits could be to amplify the signal of particular DNA bound transcriptional activators by helping target other coactivator complexes and increasing either the steady-state amount and/or induction kinetics of certain transcripts. This idea is supported by studies of *S. cerevisiae* showing the occupancy of the SAGA coactivator complex at certain promoters can be interdependent with (14) or dependent on (13) Mediator and its tail module subunits. A recent study shows that Swi/Snf activity at the *S. cerevisiae CHA1* promoter also is dependent on Mediator tail module subunits (45). Consistent with such a scenario, we have found that the TADs of *Sc*Med2 and *Sc*Med3 appear to function redundantly in the induction of high levels of activated transcription. A phenotype associated with the individual deletion of *ScMED2* or *ScMED3* is the inability to utilize galactose as a carbon source (23). This phenotype directly correlates with gene expression defects in the Δ*med2* and Δ*med3* strains (23). Individual deletion of the TAD of *ScMED2* (*MED2*Δ*C*) or *ScMED3* (*MED3*Δ*C*) does not have a pronounced effect (Fig. 4A) on this phenotype. However, deleting both TADs (*MED2*Δ*C*/*MED3*Δ*C*) does lead to a growth defect on galactose that is intermediate compared to the individual or combined deletion of *MED2* and *MED3* (Fig. 4A). Using quantitative RT-PCR, we determined that an accompanying in-

termediate defect in transcriptional induction of the *GAL* genes was present in the *S. cerevisiae* strain lacking the Med2 and Med3 TADs (Fig. 4B and C). Since previous data suggest the presence of an additional TAD in ScMed15 (8), it is possible that there is additional redundancy in the tail module that allows for these intermediate levels of induction. Mediator occupancy, in a wild-type strain, at the Gal4 binding site in the *GAL1,10* promoter increases dramatically when shifting the sole carbon source from raffinose to galactose (46). To determine whether the removal of the TADs influenced a pre- or post-Mediator occupancy step, we performed a ChIP experiment at this locus using tagged middle module (Med7)- and head module (Med17)-tagged strains (Fig. 4D and E). The overall pattern and enrichment of occupancy at the UAS observed in the wild type is preserved in the mutant. This indicates that the Med2 and Med3 TADs predominantly influence a post-Mediator occupancy step in *GAL1,10* induction. The Med17 data show that there may be a slight decrease in Mediator occupancy in the induced state. This could indicate that the TAD mediates an interaction between coactivators (14) that facilitates Mediator recruitment. Compared to that of *S. cerevisiae*, the single TAD present in the *C. dubliniensis* Tlo1 subunit has a stronger effect on the induction of a similar response.

The Mediator-associated activation domain of *C. dubliniensis* Tlo1 is required for response to carbon source and oxidative stress. Of the two *TLO* genes in *C. dubliniensis*, *TLO1* is expressed at 50-fold higher levels than *TLO2* under standard growth conditions and can complement most phenotypes associated in a strain with both deleted *TLO* genes (12). We tested the impact of the Tlo-associated TAD in *C. dubliniensis* by removing the C-terminal TAD in the only source of *TLO1*. Similar to *S. cerevisiae* and *C. albicans*, purification of the *C. dubliniensis* Mediator showed that the C-terminal TAD of Tlo1 was not necessary for its incorporation into an intact Mediator complex (Fig. 5). Complete deletion of *C. dubliniensis TLO1* and *TLO2* results in the inability to grow on galactose as its sole carbon source or under conditions of oxidative stress (12). These phenotypes are complemented by expressing full-length *TLO1* in the mutant strain (12). To test whether the C-terminal TAD of Tlo1 was important for these adaptive responses, we tried to complement these phenotypes with the truncated Tlo1. *TLO1*Δ*C* was only slightly better than the mock vector control in its ability to grow on galactose and to induce the *C. dubliniensis GAL1* and *GAL10* genes (Fig. 6A to C). In addition, *C. dubliniensis TLO1*Δ*C* also was unable to complement the oxidative stress susceptibility phenotype of the *tlo* deletion mutant (Fig. 6D). These data lead us to speculate that the TLO TADs in *C. dubliniensis* and *C. albicans* play a particularly important role in adaptive responses in these pathogens.

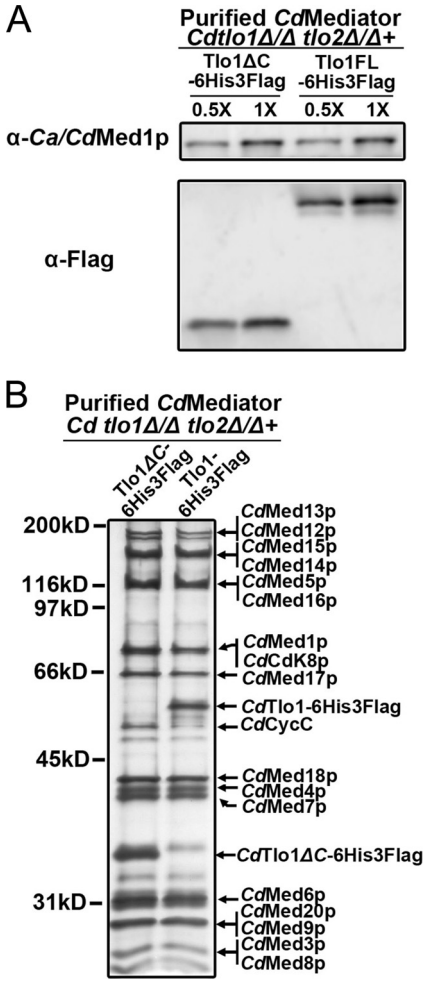

FIG 5 The N terminus of a *C. dubliniensis* Tlo1 protein is sufficient for incorporation into Mediator. (A and B) Affinity purification of Mediator from *C. dubliniensis* strains with a tag placed on the C-terminal end of full-length Tlo1 [*TLO1-6HIS-3FLAG* (yLM253)] or Tlo1Δ*C* [*TLO1*Δ*C-6HIS-3FLAG* (yLM254)] results in isolation of an intact complex as monitored by an immunoblot demonstrating equal *Cd*Med1 content (A) and by silver stain of the isolated complexes resolved by 10% SDS-PAGE (B).

DISCUSSION

This initial characterization of fungal Mediator-associated TADs could have a broad impact on the understanding of important areas in transcription regulation and fungal pathogenesis. The *S. cerevisiae* Mediator tail module largely regulates SAGA-dependent genes and helps direct the activity of the SAGA and Swi/Snf coactivator complexes to specific promoters (13, 14, 45, 47). The interaction of Mediator-associated activation domains with well-characterized targets of certain DNA-bound transcriptional activators (1), such as Tra1 (48), could explain how Mediator coordinates the recruitment of SAGA or other coactivators. Preliminary mass spectrometry experiments probing proteins associated with free *C. albicans* Tlo protein indicate that these interactions are likely to be present. Experiments in which the activation of reporter genes result from the fusion of Mediator subunits to sequence-specific DNA binding domains (6–8), or the creation of a new Mediator-DBD binding interaction (44), have been interpreted to suggest that direct recruitment of Mediator to promoters, in the absence of a TAD, led to activated transcription. A recent comprehensive study of nonclassical activators, however, showed that only *Sc*Med2 and *Sc*Med3 had the capability to strongly stimulate transcription when fused to a DBD in *S. cerevisiae* (8). Our finding that Med2/Tlo and *Sc*Med3 possess TAD activity that is separable from their incorporation into Mediator suggests that mechanisms beyond recruitment are critical for a TAD to stimulate the functionality of the complex. An interesting unanswered question remains: why does the indirect targeting of Mediator associated TADs to promoters by fusion of other Mediator subunits to DBDs fail to activate transcription (8)?

Even though there are no clear metazoan *MED2* and *MED3*

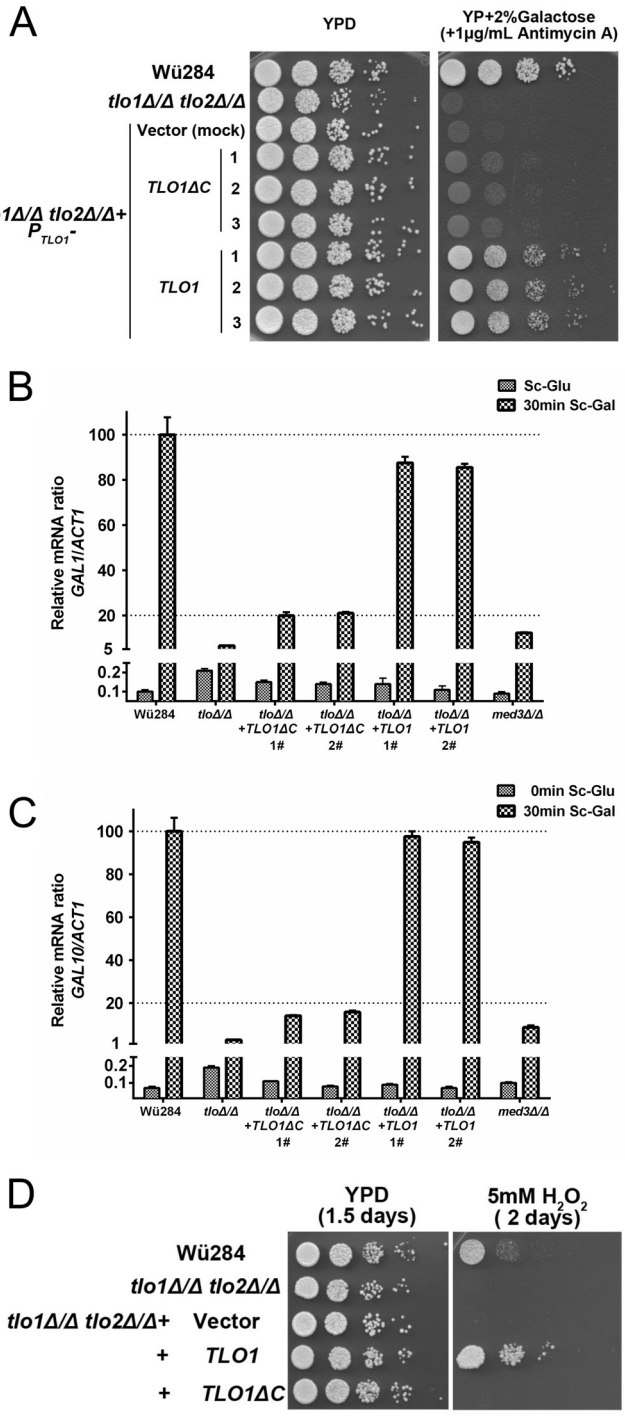

FIG 6 The C-terminal TAD of *C. dubliniensis* Tlo1 is required for growth on galactose and under conditions of oxidative stress. (A) Series dilution growth assay testing the fitness of a wild-type (Wü284) and *tlo* null strain (*tlo1*Δ/Δ *tlo2*Δ/Δ) strain complemented with the resistance marker only [Vector (mock)] and multiple independent clones complemented with a single copy of C-terminally truncated or full-length Tlo1 on YPD and YP Galactose (supplemented with 2 μg/ml AA) agar plates. (B and C) RT-qPCR comparing the activation of *GAL1* (B) and *GAL10* (C) upon galactose induction, after growth on glucose, in the strains described above (A) and a *med3*Δ/Δ strain, which also was unable to utilize galactose carbon source (12). (D) Series dilution growth assay testing the fitness of the strains depicted in panel A on YPD-agar and YPD-agar supplemented with 5 mM H_2O_2.

orthologs, Mediator-associated TADs likely are not restricted to fungi and could themselves be a target of regulation. A domain within the metazoan-specific Med25 tail module subunit appears to have a TAD domain that targets CBP (49). Interestingly, the presence of Mediator-associated TADs is associated with the targeting of *S. cerevisiae* Med2 (8), Med3 (50), and the *Arabidopsis* Med25 ortholog (51) by E3 ubiquitin ligases. There has been considerable work showing that the targeting of classical DNA-bound TADs by the ubiquitin proteasome system can potentiate or downregulate their function depending on the specific TF and context (52, 53). The targeting of Mediator-associated TADs by this system could be a way that the cell signaling directly regulates Mediator activity under certain conditions.

Lastly, a concept that is useful in envisioning how the large free population of *C. albicans* Tlo protein (9) could affect transcriptional regulation in the pathogen is "squelching." Defined as the downregulation of transcription by overexpression of a transcriptional activator, presumably by sequestering activator targets off chromatin, squelching has been demonstrated in artificial (54) and a limited number of physiological (55) systems. The transcriptional regulation of the *C. albicans* Tlo proteins in response to pathways that impact pathogenesis (56, 57) suggests a novel role for squelching in virulence gene expression, where the variation of the free Tlo pool could up- or downregulate genes, which are regulated by DNA-bound TADs that target the same coactivators. Whether Med2 and Med3 expression is regulated in *S. cerevisiae* is an open question. Recent proteomic quantification of abundance puts Med2 and Med3 on the lower end compared to other Mediator subunits (58). *MED2* and *MED3* generally are not affected in *S. cerevisiae* genome-wide mRNA expression studies under different conditions, although differences have been observed under a limited number of circumstances (59). Differences in the expression of Mediator subunits impacting pathophysiology is not unique to fungi. The expression of the human Mediator subunit, Cdk8, is increased in 70% of colorectal cancer samples and is significantly correlated with increased colon cancer-specific mortality (60).

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